The Carbon Module Labeling (CAMOLA) Technique

A Useful Tool for Identifying Transient Intermediates in the Formation of Maillard-Type Target Molecules

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ABSTRACT: Although the Maillard reaction is a well-known source of aroma and taste compounds in processed foods, a systematic correlation of the concentrations of most of the reaction products identified so far with human perception has scarcely been performed. Furthermore, the influence of process parameters on yields and formation mechanisms of key flavor compounds has not been systematically studied. In this short state-of-the-art review, concepts to characterize flavor-active food constituents are briefly discussed, and approaches to elucidate formation mechanisms from labeling experiments and isotopomeric quantitation are highlighted on the basis of results obtained in the author's laboratory.

KEYWORDS: carbon module labeling; CAMOLA; 4-hydroxy-2,5-dimethyl-3(2H) furanone; 5-acetyl-6-methyl-2,3-dihydro-1H-pyrrolizine; 7-acetyl-5-methyl-2,3-dihydro-1H-pyrrolizine

BACKGROUND

On the basis of results obtained in many studies aimed at identifying compounds formed during thermal treatment of carbohydrate/amine model systems, the so-called Maillard reaction (MR) has turned out to be a very complex reaction cascade, even if only one carbohydrate is reacted in the presence of one amino compound. Besides the important role of the MR in the generation of aroma-1 or taste-active compounds,2 compounds showing antioxidative activities3 also have recently been identified in Maillard-type reactions. In addition, food-borne toxins, such as acrylamide,4,5 or proteins modified in their amino acid side chains, the so-called advanced glycation end products like carboxymethyllysine,6 are well-known compounds formed in the MR during food processing. Because there is a growing interest in avoiding the formation of unwanted MR products but maintaining the levels of desired compounds, more detailed information on pathways leading to single “bioactive” target molecules is needed.
The use of isotopically labeled organic compounds proposed as precursors or intermediates in the formation of certain target molecules is a powerful technique to elucidate complex reaction pathways. Chemicals radiolabeled with \(^3\)H or \(^{14}\)C offer the highest sensitivity in the detection of labeled metabolites, but specific detectors or a laborious isolation of the target molecule from the matrix is needed to precisely determine the specific radioactivity. Furthermore, an important drawback of the use of radiolabeled precursors is that the number of labeled atoms in the target molecule—and their position in the entire structure—can be obtained only by retrosynthetic means, that is, a chemical degradation.

Using \(^{13}\)C or \(^2\)H labeling in the precursor compounds in combination with either mass spectrometric or nuclear magnetic resonance (NMR) measurements offers two opportunities: the number of labeled atoms incorporated in the target molecule can be determined by mass spectrometry and the “en bloc” transfer of several carbon atoms as a “module” can be derived from the \(^{13}\)C-NMR coupling patterns.

In more traditional approaches used in previous studies, an appropriately labeled precursor was reacted under Maillard-type conditions, and mostly carbohydrates labeled at carbon-1 were used. The metabolite under investigation was isolated and analyzed by mass spectrometry. An increase in the molecular mass by one unit served as proof that the label had been introduced in the target molecule. This concept, implying the validity of a single formation pathway, has been used to clarify Maillard-type reactions of, for example, isoleucine, \(^7\) proline, \(^8\) or alanine. \(^9\) Moreover, the formation of important food aroma compounds, such as 2-acetyl-1-pyrroline and 2-acetyltetrahydropyridine from proline/glucose mixtures, has been clarified. \(^10\)

However, the intermediates involved in the formation reaction cannot be derived from such data. Furthermore, it has also been shown that even when starting from one precursor, often two or more completely different pathways may run in parallel but lead to the same target molecule. \(^11,12\) One important reason is that different transient intermediates formed by degradation of the carbohydrate skeleton may be involved in the formation of the target compound.

To evaluate how many different pathways lead to a certain target molecule and to quantify the relative importance of each pathway, we recently developed the so-called CAMOLA (carbon module labeling) technique. \(^13\) In principle, this approach uses a quantitatively defined mixture of the unlabeled and the ubiquitously labeled precursor carbohydrate. The thermally induced breakdown of the carbohydrate skeleton, followed by a recombination of the intermediates formed, generates a mixture of isotopomers of the target molecule from which the importance of single reaction pathways can be deduced based on mass spectroscopic data and statistical rules. In the following, I will (i) discuss concepts to identify key aroma- and taste-active compounds and (ii) explain the CAMOLA approach based on the successful clarification of reaction pathways leading to selected “bioactive” target molecules.

**Flavor-Active Food Constituents**

The typical flavors (aroma and taste) of thermally processed foods, such as bread crust, roasted cocoa, coffee, or French fries, are caused mainly by the formation of Maillard-type reaction products. Identification of the entire set of volatiles formed during manufacturing of foods has been the focus of many previous investigations. However, in most cases human perception has not been correlated with analytical
data, and thus the “bioactivity” of compounds formed in Maillard-type reactions in terms of their aroma or taste activity remained unresolved.

To determine which compounds among the volatile food constituents make a contribution to a given food aroma, we previously developed a four-step procedure shown in FIGURE 1. In a first step, a very careful procedure, such as the SAFE (solvent-assisted flavor evaporation) technique, is applied for volatile isolation. The distillate obtained is sensorially evaluated to prove the similarity of the odor with the aroma of the food itself.

In a second step, odor-active constituents are screened by gas chromatography/olfactometry, and an approximation of the aroma contribution is done by application of the aroma extract dilution analysis. In a third step, odorants showing the highest flavor dilution (FD) factors are quantified using stable isotope dilution assays, and their concentrations are divided by their odor thresholds in the respective food matrix. Compounds exhibiting odor activity values (OAV; the ratio of concentration to odor threshold) greater than 1 are then proposed for use in an aroma recombinate based on the natural concentrations determined in the food sample. This recombinate is manufactured using reference aroma compounds. Identification and quantitation procedures are considered successful only if the overall aroma of the recombinate is in agreement with the food aroma itself.

Because typically only about 5–10% of food volatiles are aroma active, only the clarification of pathways leading to these characteristic-impact aroma compounds is
a rational basis to improve food aromas. A similar approach is used in the identification of taste-active compounds.\textsuperscript{2}

For example, the structures of the odorants identified with the highest FD factors in roasted cocoa beans are displayed in Figure 2. Among the compounds identified, 4-hydroxy-2,5-dimethyl-3(2\textit{H}) furanone (4-HDF), with an intense caramel-like aroma, was the most odor active. Quantitative measurements performed on unroasted and roasted cocoa beans from the same batch indicated a clear formation of this aroma compound during roasting (Table 1), whereas the respective 3-hydroxy-4,5-dimethyl-2(5\textit{H})-furanone was not increased, although its structure might imply the MR as the source (Fig. 2).

The compound 4-HDF has previously been identified as a contributor to the overall aromas of several foods, and a calculation of its odor activity values based on the concentrations determined confirmed the general importance of 4-HDF in food aromas (Table 2). On the other hand, the large differences in the amounts present in the

<table>
<thead>
<tr>
<th>Odorant</th>
<th>Concentration ((\mu g/kg)) Unroasted cocoa</th>
<th>Roasted cocoa</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Hydroxy-2,5-dimethyl-3(2\textit{H})-furanone</td>
<td>17</td>
<td>990</td>
</tr>
<tr>
<td>3-Hydroxy-4,5-dimethyl-2(5\textit{H})-furanone</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>3-Methylbutanal</td>
<td>1100</td>
<td>26,400</td>
</tr>
<tr>
<td>3-Methylbutanoic acid</td>
<td>14,200</td>
<td>17,300</td>
</tr>
</tbody>
</table>

FIGURE 2. Key aroma compounds (FD \(\geq\) 512) identified in roasted cocoa beans. (Modified from Frauendorfer & Schieberle.\textsuperscript{20})
different foods suggest either that the concentrations of its precursors in the raw material are different or that different pathways running with different yields are operating. The latter suggestion is quite obvious, because malt, for example, contains the highest amount of reducing carbohydrates, but during the malting process only a comparatively low amount of 4-HDF is formed (TABLE 2).

**Formation Pathways Leading to 4-Hydroxy-2,5-Dimethyl-3(2H)-Furanone**

Besides rhamnose as an important precursor, we could previously demonstrate\textsuperscript{16} that 4-HDF can be formed by a reduction of acetylformoin, as shown in FIGURE 3. This reduction may occur either in a disproportionation reaction as described in the work of Schieberle\textsuperscript{16} or by a Strecker reaction with amino acids (e.g., proline).\textsuperscript{11}

### TABLE 2. Concentrations and odor activity values of 4-hydroxy-2,5-dimethyl-3(2H)-furanone (4-HDF) in several foods

<table>
<thead>
<tr>
<th>Food</th>
<th>Concentration (µg/kg)</th>
<th>OAV\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coffee</td>
<td>131,000</td>
<td>2183</td>
</tr>
<tr>
<td>Cooked beef</td>
<td>9075</td>
<td>151</td>
</tr>
<tr>
<td>Rye bread crust</td>
<td>4310</td>
<td>72</td>
</tr>
<tr>
<td>French fries</td>
<td>2591</td>
<td>43</td>
</tr>
<tr>
<td>Wheat bread crust</td>
<td>1920</td>
<td>32</td>
</tr>
<tr>
<td>Popcorn</td>
<td>1370</td>
<td>23</td>
</tr>
<tr>
<td>Roasted cocoa</td>
<td>990</td>
<td>17</td>
</tr>
<tr>
<td>Barley malt</td>
<td>820</td>
<td>14</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Odor activity values were calculated by dividing the concentration by the odor threshold in cellulose as model matrix.

![Chemical structures](image)

**FIGURE 3.** Formation of 4-hydroxy-2,5-dimethyl-3(2H)-furanone from glucose via a reduction of acetylformoin as the key transient intermediate.
However, in both cases the carbohydrate skeleton should remain intact in the 4-HDF generated. On the other hand, the carbohydrate may be cleaved to form reactive transient intermediates, which in turn recombine to generate 4-HDF.

To get a clear insight into the importance of the formation pathways, a 1:1 mixture of $[^{12}\text{C}]_6$glucose and $[^{13}\text{C}]_6$glucose was reacted with L-proline under low-water conditions as well as in aqueous solution, and the isotopomeric pattern in the 4-HDF formed was analyzed by GC/MS. The application of this CAMOLA approach would show the following (Fig. 4): If the carbohydrate skeleton remains intact during 4-HDF formation, a 1:1 mixture of $[^{12}\text{C}]_6$4-HDF and $[^{13}\text{C}]_6$4-HDF should be obtained. But if a breakdown of the carbohydrate occurs before 4-HDF is formed, five other isotopomers ($[^{13}\text{C}]_3$4-HDF to $[^{13}\text{C}]_5$4-HDF) may be generated.

The results obtained were quite challenging: When the mixture was reacted under roasting conditions (low water content), a 1:1 mixture of $[^{12}\text{C}]_6$4-HDF and $[^{13}\text{C}]_6$4-HDF was formed (Fig. 5a). But when the reaction was performed in an aqueous buffer in an autoclave at 145°C, $[^{13}\text{C}]_3$4-HDF was formed as the main isotopomer. This isotopomer was present only as a very minor compound at low-water conditions (Fig. 5a). Because, for statistical reasons, $m/z$ 131 representing the $[^{13}\text{C}_3]$ isotopomer should be present in double the intensity compared with $m/z$ 128 ($[^{12}\text{C}_6]$4-HDF) or $m/z$ 134 ($[^{13}\text{C}_6]$4-HDF), it could be calculated that about 60% of the 4-HDF was formed by a recombination of two C-3 fragments.
FIGURE 5. (A) Mass spectrum of 4-hydroxy-2,5-dimethyl-3(2\(H\))-furanone (4-HDF) formed from glucose and \(^{13}\text{C}_6\)glucose (1+1) when reacted with proline under roasting conditions (15 min at 160°C; 10% water). (B) Mass spectrum of 4-hydroxy-2,5-dimethyl-3(2\(H\))-furanone (4-HDF) formed from glucose and \(^{13}\text{C}_6\)glucose (1+1) when reacted with proline in aqueous buffer (145°C; 20 min).
A possible formation pathway is suggested in Figure 6. Hydroxyacetone, a well-known degradation product of carbohydrates, may tautomerize into an endiol, which by a reaction with 2-oxopropanal forms 2,5-dioxo-3,4-dihydroxyhexane. Its cyclization, followed by β-elimination of water, finally yields 4-HDF. Because both C-3 intermediates may be labeled, this intermediate is statistically present in double the amount. To prove this assumption, both C-3 intermediates were reacted in aqueous buffer and the amounts of 4-HDF formed were quantified. The results (Table 3) indicated that these intermediates can form substantial amounts of 4-HDF. Because the formation was drastically lowered at pH 3.0 than at pH 7.0, a retro Aldol reaction, as shown in Figure 6, is most probable. Recently, we could show that the carbohydrate breakdown is much more favored when high hydrostatic pressure is applied in Maillard-type reactions. 17

**Formation Pathways Leading to Bitter-Tasting Dihydro-1H-Pyrrolizines**

Besides the well-known diketopiperazines, which are predominantly formed by a condensation of two amino acids in the absence of carbohydrates, only a few other compounds eliciting bitter taste have been identified in Maillard-type reactions. 21–23 Bitter-tasting compounds are assumed to be formed predominantly from carbohydrates in the presence of proline, and the structures of four compounds previously

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**TABLE 3. Influence of pH on the amounts of 4-hydroxy-2,5-dimethyl-3(2H)-furanone (4-HDF) formed from 2-oxopropanol and hydroxyacetone**

<table>
<thead>
<tr>
<th>pH</th>
<th>µg</th>
<th>mol-%</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>59.6</td>
<td>0.05</td>
</tr>
<tr>
<td>5.0</td>
<td>364.5</td>
<td>0.28</td>
</tr>
<tr>
<td>7.0</td>
<td>1450.5</td>
<td>1.10</td>
</tr>
</tbody>
</table>

*Data from Frauendorfer & Schieberle.* 20

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**FIGURE 6.** Formation of 4-hydroxy-2,5-dimethyl-3(2H)-furanone from the transient intermediates hydroxyacetone and 2-oxopropanal.
suggested to contribute to the bitter taste are displayed in Figure 7. In particular, the structural similarity of the 5-acetyl-6-methyl-(5-AMDP) and the 7-acetyl-5-methyl-2,3-dihydro-1H-pyrrolizine (7-AMDP) prompted us to investigate whether similar mechanisms are involved in the formation of both compounds. Details on the experiments will be published elsewhere.18

For this purpose, I again reacted a 1:1 mixture of $^{12}$C$_6$ glucose and $^{13}$C$_6$ glucose in the presence of proline in phosphate-buffered saline (145°C; 1 h; autoclave) and quantified the amounts of 5-AMDP and 7-AMDP formed. The results showed that predominantly the 5-acetyl derivative was formed, and the amounts were higher than those of the corresponding 7-acetyl derivative by a factor of 34. Mass spectrometric analysis of the isotopomeric composition showed a very interesting result: The 5-AMDP was generated as mixture of three isotopomers at $m/z$ 163, $m/z$ 166, and $m/z$ 169 in the ratios 1:2:1, thereby indicating a formation involving transient intermediates with three carbon atoms (Fig. 8a). However, the 7-AMDP consisted of four isotopomers in the ratios 1:1:1:1 at $m/z$ 163 ($^{13}$C$_0$7-AMDP), $m/z$ 165 ($^{13}$C$_2$7-AMDP), $m/z$ 167 ($^{13}$C$_4$7-AMDP), and $m/z$ 169 ($^{13}$C$_6$7-AMDP). For statistical reasons, these results implied that two transient intermediates with two and four carbon atoms are involved in the formation of 7-AMDP.

To check their efficacy in forming 5-AMDP, I reacted several C-3 compounds and mixtures thereof in the presence of proline (150°C; 1 h; autoclave) and quantified the amounts of 5-AMDP formed. The results indicated a 1:1 mixture of glyceraldehyde and hydroxyacetone as the most effective precursor mixture. When reacted with proline, these compounds formed more 5-AMDP than glucose by a factor of 30 (data not shown).

A possible route leading to this bitter tastant is shown in Figure 9. In a first step, proline reacts with glyceraldehydes, thereby forming the Amadori compound. This intermediate may eliminate water and, after elimination of carbon dioxide, reacts

FIGURE 7. Bitter-tasting compounds identified in a glucose–proline mixture reacted at 150°C for 1 h under aqueous conditions.
FIGURE 8. (A) Mass spectrum of 5-acetyl-6-methyl-2,3-dihydro-1H-pyrrolizine (5-AMDP) formed from proline in the presence of a 1:1 mixture of glucose and $[^{13}C_6]$glucose. (B) Mass spectrum of 7-acetyl-5-methyl-2,3-dihydro-1H-pyrrolizine (7-AMDP) formed from proline in the presence of a 1:1 mixture of glucose and $[^{13}C_6]$glucose.
FIGURE 9. Suggested formation pathway leading to 5-acetyl-6-methyl-2,3-dihydro-1H-pyrrolizine (5-AMDP) from proline, glyceraldehydes, and hydroxyacetone.

FIGURE 10. Suggested formation pathway leading to 7-acetyl-5-methyl-2,3-dihydro-1H-pyrrolizine (7-AMDP) from proline, the 1-deoxytetrosone (C4), and acetaldehyde (C2).
with hydroxyacetone to yield a di-substituted pyrroline. Cyclization and elimination of water finally yields the 5-AMDP.

Model studies on carbohydrate degradation products yielding higher amounts of 7-AMDP indicated that a mixture of D-erythrose and acetaldehyde was most effective by increasing the yield by a factor of 20 compared with the glucose–proline reaction.

A possible formation pathway of 7-ADMP from erythrose, proline, and acetaldehyde is proposed in Figure 10. The 1-deoxyosone of erythrose might form an imine with proline. Decarboxylation of this intermediate in a Strecker-type reaction generates an N-substituted 1-pyrroline, which can cyclize with carbon 2 of the pyrroline ring. The bicyclic endiol formed can react with electrophilic compounds, such as acetaldehyde, and the intermediate formed rearranges into the stable pyrrol system of 7-ADMP by elimination of water.

In the meantime, the CAMOLA approach has also been applied in the clarification of pathways governing the formation of posttranslational protein modifications, such as carboxymethyllysine (see Kasper and Schieberle,19 this volume), the results of which will soon be reported.

REFERENCES

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